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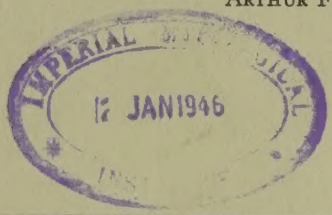

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THE BOTANICAL REVIEW

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QUANTITATIVE BIOASSAY OF FUNGICIDES IN THE LABORATORY

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INTRODUCTION

Some unkind wag has said that if all the scientists of the world were placed end to end they would never reach a conclusion. Although scientists resent this statement most bitterly, it is true enough to get a laugh. Its kernel of truth lies in the fact that nature is so complex that she presents a different face to nearly every experimenter. Nature could not present so many faces if the cameras of the experimenters had wide-angle lenses or if they were set up in enough places to cover all of nature's poses.

It is the purpose here to set down and to evaluate critically the progress that has been made in designing proper equipment to reach conclusions regarding that little segment of nature known as fungicidal action. The discussion will be limited primarily to the laboratory techniques that have been devised for testing chemicals. The action of heat will be omitted.

A few years ago it was customary to take a dim view of accelerated techniques of assaying fungicides, probably because results from them unhappily would not check with field results, or even with themselves. The first criticism was hardly justified because field results seldom checked themselves. Martin (68) has well said that "The extent to which the laboratory trial will be confirmed by field trial will be controlled by the correctness of the allowances made for the influence of the missing factors in the interpretation of the results". During the time since Martin's paper was published, so many "missing factors" have come under control that we now think that if a compound does not work in the field when it does work in the laboratory, the field technique should be questioned before the laboratory technique.

The discussion will be limited primarily also to bioassay techniques because, as yet, chemical techniques have not caught up with bio-techniques. Fungi are much more vocal in expressing their preferences or rather dislikes for different fungicides than known chemical reagents have been. When the chemists will have polished up the techniques for fungicide assay, everyone will gladly turn to them, but until such time bioassay must lead the way.

The word fungicide will be used here in the largest sense, meaning either to kill or inhibit a fungus, or to prevent its effects.

Although laboratory bioassay was used as early as 1807 by Prévost (87) in his classical researches on fungicidal action of copper, the technique has languished unduly. Happily this situation is changing with the realization that rapid further progress in the art must await consolidation of the science.

OBJECTIVES IN THE BIOASSAY OF FUNGICIDES

The techniques of assay, of course, must depend largely upon the objectives that are in mind. Some are interested in discovering a new fungicide. They would probably prefer, first, a screening technique that would throw out unlikely materials.. In our laboratory we have put some 6,000 compounds through such a set of screens of ever growing fineness. The screenings will, of course, be governed by the screen used. One can never be sure that what he throws away as chaff may not contain some grain. One company in screening a series of materials as fungicides discarded tetrachloroquinone (Spergon), but a different screen sorted it out. Such instances have probably occurred before and will occur again. Nevertheless, a screen must be used because most of the material thrown out will be chaff if the screen is reasonably well designed.

Secondly, those interested in developing new materials desire speed. They cannot test several thousand compounds a year in the field, but they can in the laboratory.

Thirdly, they need a method of quality control after they succeed in isolating a new compound because large scale production may have difficulties not encountered in the laboratory where production was in beakers.

One of the limiting factors in screen tests is the number of

organisms that can be used as guinea pigs. Therefore, those interested in fungicides to control specific organisms or mixtures of organisms must test with these. Sometimes different tests must be devised to fit the idiosyncrasies of the organisms.

Moreover, specific uses necessitate other specific tests. Does the material dust well or suspend well in a spray tank? Does it react with other ingredients in the tank? Will it penetrate wood or fabric? Is it volatile at high temperature? Is it toxic to animals?

A few people are interested in the nature of fungicidal action. How do fungicides produce their effect on fungi? Specific techniques may be needed here.

Finally, the farmer, the army or the railroad may like to know how well a material will perform in preventing a plant disease, or in protecting a tent or a railroad tie from decomposing. This last, of course, is a field test and off the reservation for us. We shall attempt to cover the major techniques that apply to the other objectives.

RÔLE OF FUNGICIDES IN CONTROL OF FUNGI

If the derivations of assay techniques are to be grasped, then the uses for fungicides must be reviewed briefly. The time has passed when one can expect a simple overall test such as spore germination to cover the practical uses for fungicides. Chemical fungicides may perform two basic functions in the art of fungus control, prophylaxis and chemotherapy. The purpose of prophylaxis is to protect an object such as a leaf, the human skin or a tent against attack. The object of therapy is to cure an active infection after it has been established. When this is done with chemicals, it is chemotherapy. Obviously, basically different assays will be needed for each. Most assays so far developed apply in the field of prophylaxis or protection. Since many aspects of chemotherapy are new, few techniques are available in that field.

PROPHYLAXIS

Perhaps 99% of current tonnage of fungicides is used for protecting something against fungi. The major portion of this tonnage is applied directly to the object to be protected. Wood is "doped" with creosote to protect it against decay; fabrics are

dunked in copper solutions to make them "mildew proof"; and untold acres of crops are sprayed or dusted until the cuticle is worn thin to protect them against scab, rust, blights and rots of all description. Fungi in their life cycles find themselves open to poisoning at one or both of two points—before inoculation can occur or after inoculation but before penetration.

Preventing inoculation. Despite possible noises from kibitzers, the word "inoculation" will be used here to cover fence posts as well as wheat plants. No other word seems to cover the necessary concepts involved. If chemicals are to prevent inoculation they must be applied to the source of inoculum, whether it be somewhere in the environment of or on the previous host. They may act by killing or inhibiting the fungus or by preventing its sporulation.

Preventing infection after inoculation. Fungicides have played a much wider rôle in preventing infection after inoculation than they have in preventing inoculation.

The majority of protective chemicals are applied to an infection court before inoculation, even though, obviously, they can not act until inoculation has occurred. Wood is creosoted and an apple tree is sprayed before inoculation. In some cases, however, treatment is not applied until after inoculation. Outstanding examples in this field are treatment of wheat seed contaminated with bunt and treatment of contaminated peach buds to control peach leaf curl. In both cases the plant is protected from *infection* even though *inoculation* may already have occurred.

A very special type of prophylaxis merits description. Protectants are now being placed inside a plant rather than outside. Perhaps this should be labelled as "artificial immunization," but, taking the plant's-eye view, it is prophylaxis. Zentmyer (125) has shown quite recently that a small percentage of seedling elms may be thus protected against *Ceratostomella ulmi,* and Stoddard (102) has just shown that peach trees can be protected against the X-disease virus by injecting chemicals into the plants. Clayton *et al.* (13) have suggested that certain glyceride oils appear to immunize tobacco against the downy mildew fungus, but that they do not prevent spore germination. Hence a spore inhibition technique would not have screened out this useful fungicide for that disease.

CHEMOTHERAPY

Although chemical prophylaxis has dominated the field in late years, chemotherapy without doubt was the earliest type of control tried. This was due, of course, to the desire to "treat" a sick plant. One did not worry about plants that were well.

External therapy of powdery mildews with sulfur is probably the oldest practice in plant pathology. It is equally old in human pathology. The use of juglone (5-hydroxy-1-4 naphthoquinone) in walnut husks for fungus ring worm on the skin goes back at least to colonial times.

Internal use of fungicides in plants to alleviate infections is new. Its modern revival as a bona-fide procedure and not a mumbling of quacks probably dates from the work of Howard (47). The Germans have talked much of the "chemotherapeutical index" in their work on cereal seed treatments. Riehm (92) castigates them for the term by pointing out that they were dealing with prophylaxis and not with therapy.

MECHANISMS OF FUNGICIDAL ACTION

Development of assay techniques must be based on the mode of fungicidal action as well as on the uses to which the fungicide is to be put. Four mechanisms of fungicidal action have been uncovered so far. A fungicide may prevent sporulation. Churchman (12) has defined this as "genestatic" because it keeps genesis static. The genestatic properties, of course, can be studied best in culture. Fungicides may inactivate mycotoxins and thus reduce the pathogenic action of the fungus, as Howard has shown (47). This power can be measured in the laboratory on extracts from the fungus, as has been shown (125) for toxins from the Dutch elm disease fungus.

The second two phases of fungicidal action, true killing and inhibition of the fungus, are exceedingly difficult to separate in practice. Usually the two concepts are distinguished by saying that the former is fungicidal action in the sense of the strictest definition and that the latter is fungistatic action. Of course, to the practitioner of the art, it is immaterial whether the fungus is killed or not, but it is of considerable theoretical significance and it often has practical value.

To distinguish between fungicidal and fungistatic, the treated

fungus is placed in a poison-free environment. If it grows again, the action can be assumed to be fungistatic. Time, of course, is a factor (58) in fungistatic value. The longer the fungus is allowed to remain in contact with the fungicide, the less its likelihood, in general, of recovering when placed in a poison-free environment.

Protectants, by definition, must be applied to and remain in the infection courts. They must, therefore, be insoluble in water and have other qualities of resistance to weathering if they are not to be removed before thay can fulfill their function. Horsfall, Marsh, and Martin (46) have divided protective action into the dose factors which determine quantitatively the amount and distribution of the chemical, and the factors for fungicidal value which determine qualitatively the ability of the chemical to inhibit the fungus.

Dose factors can be studied in the laboratory as exercises in physics. They are concerned with deposition, retention, adherence and tenacity, all problems in logistics, to use a current military idiom. They involve getting the materials to the proper place at the proper time.

The factors in fungicidal value can be studied in the laboratory as exercises in chemistry. They are concerned with availability, *i.e.*, the speed with which killing concentrations can be extracted from the insoluble residue. They are also concerned with imherent toxicity, *i.e.*, the ability of the solubilized toxicant to kill or inhibit the fungus.

ASSAYING THE PHYSICAL FACTORS IN FUNGICIDAL ACTION

Clearly a fungicide must be offered to a fungus if it is to kill it. This is not as easy as it seems. Many failures of fungicides result from failure to reach a vital part of the fungus with a disabling dose. As the old proverb goes, "There's many a slip 'twixt the cup and the lip".

In dealing with the physical factors one must consider treatment of a surface as of a leaf or a porous solid such as soil or a piece of wood. As Martin (69, 70) has suggested, many dose factors can be determined by physico-chemical methods, but they are also amenable to bio-assay.

Treatment of Surfaces

Farmers generally treat surfaces. They spray apple foliage and dust wheat seeds. Practical men have a habit of saying that apple scab, for example, is controlled by spraying with sulfur at the rate of 5 pounds per 100 gallons. Apple scab is not controlled by the mixture in the spray tank. They forget the "slips 'twixt cup and lip". They forget the masking effects of foliage, the run-off or the drain-off. Apple scab is controlled by the sulfur that lies on the few square microns of leaf where each spore falls. The problems of placing sulfur on that area and keeping it there are many and varied, but they must be reckoned with in an assay technique.

The three physical factors that govern the effective dosage on a surface at any time are deposition, coverage and tenacity. Deposition is the process of applying the fungicide. Coverage is the uniformity and completeness of distribution of the fungicide over all the areas to be treated. Coverage is often loosely used in the sense of deposition, but it should specifically refer to uniformity and completeness of distribution. Tenacity is the factor that is of peculiar importance to protection. Tenacity is the property of a fungicide to resist removal by weathering (2). Weathering is considered to be any climatological factor operating to reduce a deposit. If the fungicide does not resist strongly the subtractive effects of weathering on the dose, it does not succeed as a protectant.

Choosing the "standard" surface. As yet no one has been able to mimic precisely a leaf surface in the laboratory. Neither has such a necessity been demonstrated. A reproducible surface is more necessary than a so-called natural surface. Some (67) have used leaves as surfaces for germinating spores, but this technique is dreadfully laborious. Others (64, 120) seem to prefer plain glass. The glass surface, however, is not very reproducible. Apparently its surface tension varies widely. Evans and Martin (18) have suggested cellulose nitrate on glass because it is reproducible. This surface has been widely used.

Deposition. Four procedures suggest themselves for applying toxicants to surfaces—pipetting, dipping or spraying the liquids and dusting the powders. If the toxicant is water-soluble, it may be pipetted directly to a surface. Inasmuch as different materials

may affect differently the interfacial tension between water and the surface, they will cover different areas and thus introduce an error. Montgomery and Moore (72) etched circles on glass to prevent spread of the drops, but that does not work too well. To prevent unequal spread of drops, Peterson (83) cemented microscope cover slips to glass and applied his materials to these. Cavity slides are useful for the purpose, but if the material is allowed to dry, it may be concentrated at the edge of the cavity by the meniscus effect. If a spore suspension is used, it may be added directly to the fungicidal liquid in the cavity slide in such volume that it just fills the cavity. This will obviate the optical difficulties of the usual drop of spore suspension and it will often eliminate the running of spores to the center of the drop. A micro-pipette or a graduated hypodermic syringe makes a good applicator.

Dipping has not proved suitable as a means of deposition primarily because of the effects of surface tension. Some materials cling better than others to the dipped surface and hence they will show more potency than the fungicidal properties warrant.

Spraying has been almost universally used as a means of depositing liquids on surfaces. Two basic types of sprayers have been used—horizontal sprayers aimed directly at the target and settling towers. In general, air-operated atomizers, sometimes euphemistically called "air brushes", are used to deposit the materials, although occasionally hydraulic sprayers similar to field models are used. Frear (26) has compared the two as horizontal sprayers and found that the atomizer gives more reproducible data than the other. He offered no explanation for this curious phenomenon.

The target in horizontal sprayers has been variously arranged in order to produce different levels of deposition. Frear (26) put the target on a large revolving wheel which passed it in front of his atomizer. He varied deposit by varying the number of revolutions. Hockenyos and Erwin (40) placed a pendulum shutter in front of a stationary target so that they could vary the deposition by varying the number of "exposures". Evans and Martin (18) used a similar apparatus. Horsfall, Marsh and Martin (46) used a guillotine type of shutter in front of the nozzle. McCallan and Wilcoxon (64) used a shut-off in the air line to regulate spray time.

Since the atomizer depends on a column of moving air to carry the spray droplets, the stream is soft. It wavers in the laboratory breezes and tends to be turbulent. Moreover, the spray fluid tends to be evaporated in transit, not only by contact with dry laboratory air at the edges of the column but also by the dry air used to drive the column. Part of these drawbacks have been avoided by placing the whole affair in a humidified hood (41). Recently a modification has been added in that the compressed air is bubbled through a washing tower. The spray stream is preferably enclosed in a tube to prevent wavering by outside breezes. Probably, however, the tube increases turbulence (86). If so, additional attention should be given to this factor.

It has been demonstrated (112) that spray droplets accumulate an electric charge as they are formed at the nozzle. Moreover, they charge the tube that they are sprayed through, and this produces variation in deposit. If, however, the tube is made of metal and grounded, the charge is drained away and the reproducibility of deposits is improved (86).

The settling tower is a standard tool in some laboratories (64). It was probably first used for insecticide work (104), and has been claimed more precise than a horizontal sprayer (64). It takes much more time, however, because each of the multiple doses must be weighed, and sprayed separately rather than varied through spray time.

Horizontal laboratory dusters have never been designed to equal the precision of horizontal sprayers, but the settling tower type (32, 38) has given good results. The size of the deposit is governed by the size of the load placed in a specially designed narrowly funneled cartridge. The charge is blown upward into the tower by means of compressed air. The settling rate of the dust follows the logarithm of time.

Coverage. Coverage, as already defined, is the uniformity and completeness of deposit, not magnitude of deposit. A deposit can be large but need not necessarily be well distributed. Presumably the best coverage obtainable of any deposit is random distribution of the particles thereof. Dusts distribute themselves over a surface at random according to the Poisson distribution (118). Sprays, however, do not give random coverage. They give blotchy coverage because the particles of toxicant travel, not in-

dependently, but in droplets. As yet no one has developed a chemical assay for coverage. One has had to be content with visual observation or photographs of the uniformity of distribution of deposits. Recently a bioassay of coverage (45) has been developed.

It appears that application of experimental deposits by spraying must be used with discretion. A given mean deposit per unit area will show more potency if applied in weak concentration for a long time than if applied in strong concentration for a short time (45). This fact has shown up only recently as technique has improved.

Surface tension of the spray fluid and of the surface affect coverage. Normally capillary-active materials are added to spray fluids to reduce surface tension and thus to improve coverage. Few realize that opposing factors operate in using such materials. It is true that any volume of liquid containing a surface-active ingredient will spread over more surface than one without. It is equally true, however, that the same ingredient will reduce the size of drops formed by and emitted from the sprayer. In fact, one of the common measures of the effect of a soapy substance on surface tension of a liquid is to count the number of drops formed as one milliliter of liquid issues from a pipette.

The small spray droplets of the liquid with a soapy ingredient will travel more slowly to a target than the larger drops of untreated liquid, and hence the deposition per unit of time will be reduced.

It follows, therefore, that the final coverage obtained on a sprayed surface will be the resultant of three forces which will vary with the surface-active ingredient used—the spreading action of the liquid over the surface, the size of the droplet that arrives on the surface and the number of droplets that arrive per unit of spraying time. Obviously, the interaction of these three factors may bias the result from two materials which differ in the amount or quality of surface-active ingredient.

If the surface tension of two spray fluids being compared differs, the results may be biased in another fashion. The drops of spore suspension applied to the sprayed surface will spread to different sizes and expose different amounts of toxicant to the spores, thus altering the results.

Adherence and retention. The magnitude of the initial deposit depends upon the property of the fungicide to adhere to any surface and upon the property of the surface to retain any material.

This matter is seriously confused in the literature for three reasons: (*a*) because initial adherence is not clearly separated from resistance to weathering, (*b*) because the property of the fungicide to cling has not always been distinguished from the property of the surface to hold, and (*c*) because our language seems to have too few available verbs. Latin provides us with two verbs, "adhaereo," to cling, and "reteneo," to hold back. From the first verb we get "adherence" and "adhesiveness." From the second we get "retention" and "tenacity." These four nouns have been used almost synonymously, or at least each has been applied to all concepts (2, 18, 21, 26, 46). Despite an earlier paper (66), somewhat at variance, it is now proposed that "adherence" (from "adhaereo") apply to the property of the fungicide to cling to the surface, and "retention" (from "reteneo") apply to the property of the surface to hold the material.

These two factors are not to be confused with resistance to weathering which is defined as tenacity (which also comes from "teneo" but which has acquired a connotation of resistance to removal rather than simple ability to cling). Fajans and Martin (21) have used "retention" to apply to the sum of adherence and retention. Frear (26) used "retention" to cover resistance to weathering or tenacity.

Since the concepts have not been too clear, little has been done in the way of devising techniques to separate them. Theoretically a surface is required to measure adherence and a fungicide is required to measure retention. Actually both are probably required for each. Usually the ultimate deposit has been measured. The difference between fungicides can be appraised by using a constant surface, and the difference between surfaces can be appraised by holding the fungicide constant.

Hilgendorff (39) analyzed wheat grain for toxicants present after treatment and showed that wrinkled seeds retain less dust than smooth seeds and that small particles of copper carbonate adhere better than large particles. Fitzgibbon (25) treated seeds with a known weight of material, dropped them through a long

glass tube onto a sieve and weighed them again to determine how much was retained.

The adherence of dust to surfaces seems to be related to electrostatic charges which Bobkov (9) has attempted to measure.

Tenacity. The factors that cause diminution in size of protective deposits are growth of the surface, scrubbing and leaching action of rain, base exchange between dust or dirt and the deposit, and sun action. Dusts may be jarred off or blown off by wind. Exceedingly little has been done on any weather factor except water. Probably it is the most important.

Tenacity, being a quantitative dosage factor, can be assayed by chemical means, provided a reagent is known for the fungicide used and provided interference with other ingredients is not serious. In such cases bioassay will be necessary. Martin (70) has said that no one has shown that bioassay of tenacity of protectants produces any more useful results than chemical assay. This is doubtless true for different samples of the same material, but Miller (71) has shown that comparisons of the tenacity of "fixed copper" materials are made more accurately through bioassay than through chemical assay.

A few chemical determinations have been made of residues on growing apple fruits. The data are inconclusive. It would seem interesting to treat a flexible surface like thin rubber and then stretch it to see what changes in fungicidal action would be induced. This would then probably turn out to be a study in coverage.

Although few studies on base exchange have been made, it has been shown here that some soils do affect fungicidal deposits, especially deposits of the relatively unstable organic fungicides such as disodium ethylene bisdithiocarbamate. It may be that low-growing plants accumulate enough dirt on their leaves to affect seriously the performance of fungicides. Tetrachloroquinone on seeds tends to be inactivated in alkaline soils. Cuprous oxide on seeds, on the contrary, tends to be solubilized in acid soils so that the injury factor is increased.

Kraus (51, 52) approximated a measure of the effect of soil on the action of protectants by germinating spores of *Tilletia tritici* on slate dust saturated with lime water. Tornow (107) claimed that the refractory germ tubes of the fungus show up

clearly against the dark soil, especially if talc is mixed with the soil.

Sunlight produces photochemical effects on many chemicals. As far as known now, it has little effect on deposits of Bordeaux mixture, but it will no doubt prove increasingly important as more organics are introduced into practice. Tetrachloroquinone already has failed as a foliage fungicide on account of photochemical effects, even though it has succeeded very well as a seed protectant when used in the dark below ground.

Some fungicides do volatilize slowly and hence lose potency slowly. Sulfur sublimes on the foliage, and the chlorinated phenols volatilize from wood and cloth. Thorburn and Vincent (106) suggested that treated fabrics be placed in front of a fan for seven days and then assayed with the test fungus. As far as known, no one has ever made similar tests on sulfur, but it needs doing.

The effects of rain on fungicidal deposits have been studied extensively since the days of Girard (30) 50 years ago. He used artificial rain on sprayed foliage. Some tests are based on simple soaking of the deposits in water. The Heuberger (37, 121) technique is more. It involves passing the treated surfaces rapidly through water to simulate the thrashing and soaking action of rains. Study of some of his data has shown, as expected, that the deposits come off in accordance with the logarithm of the number of times they are washed. From that the slope of the wash-off curve will serve as a criterion of resistance to rainfall. It serves much better for precision work than the proportion of deposit removed by a constant amount of washing, which is the usual standard. Mechanical washers (72) have been devised, and they are probably to be preferred to Heuberger's hand test. Sometimes (118) a rain test using water from a suspended nozzle has been used, but it probably is not sufficiently different in action and is very wasteful of distilled water.

Nikitin (77) felt that he could measure in the laboratory the resistance to weathering of copper protectants by measuring their adherence to the diaphragm in an electrodialysis cell.

Eidman and Berwig (17) showed that the loss of dusts by wind action seemed to follow a different course from loss by jarring and that resistance to rain seemed to follow still a different course. This suggests that the factors that govern each

differ. Doubtless, electrostatic charges are involved in prevention of loss by jarring, whereas they would play little part in loss by rain action.

Impregnation of Solids

Solids often must be impregnated with fungicides, and methods for accomplishing this are available. The solids usually concerned are woods, fabrics and soil, and the general techniques are dipping or soaking and vacuum and/or pressure.

Dipping or soaking. Perhaps the best example of soaking is the bath treatment of fabrics, the laboratory examination of which has been discussed recently (28). In another type of test the fabric is sprayed with the chemical to be tested (106).

Vacuum and/or pressure. Waterman *et al.* (113) have described a laboratory process for impregnating experimental wood blocks. They are placed in a container under a bell jar which is then exhausted to 2 cm. of mercury. Enough liquid is next introduced into the container so that the blocks are submerged when air is admitted. In commercial practice the vacuum treatment may be followed by or substituted for pressure.

Fumigation

Fumigation is sometimes used to introduce gases into solids such as the interior of a plant or soil. Techniques for evaluating fumigants have been described (20, 84, 85, 111). Entomologists also have worked extensively to develop procedures for measuring toxicity of fumigants.

ASSAYING THE CHEMICAL FACTORS IN FUNGICIDAL ACTION

Having examined the methods for applying fungicides, it is time to examine the methods for measuring the potency of the chemicals applied. Such methods range all the way from big field tests with large commercial equipment down to micropipettes and cavity slides for a microscope.

Some five laboratory or greenhouse tests have been described that give more or less information about fungicidal potency. Those adapted to the laboratory are spore germination, electrical resistance, and growth on agar, wood blocks or fabrics. Those adapted to the greenhouse are soil burial tests and infection of host plants.

Spore-germination Test

The spore-germination test may be conducted in two ways—on natural surfaces or on artificial surfaces. Tests on natural surfaces have been developed only lately and are not very useful because of the difficulty of seeing the spores. Marsh (67) germinated spores on treated apple leaves and then cleared the color from the leaves for microscopic examination. Zade (124) inoculated oat seeds with *Ustilago avenae,* treated the seeds with chemicals, removed the glumes and examined the adhering spores for germination.

The spore-germination test on artificial surfaces has been heavily investigated. Prévost (37), the pioneer laboratory researcher, used it. Carleton (11), 50 years ago, used the spore-germination test as a screen for a large number of possible toxicants for cereal rust, and Swingle (103) used it in one of the earliest studies ever made on the mechanism of action of bordeaux mixture. Although many laboratories have dabbled with it, the laboratory of plant pathology at Cornell has kept everlastingly at it under the direction of the late Professor H. H. Whetzel (116). Reddick and Wallace (89) started the work in 1910. Later workers, especially McCallan (56), carried the ball during years when laboratory assay of fungicides was in low repute. In recent years work in England (46, 67, 72) has contributed to the perfection of the technique of assay by spore germination. German workers do not seem to have paid much attention to Schmidt (95-98) who 20 years ago published rather complete techniques for laboratory assay of fungicides by the spore-germination technique.

The Committee of the American Phytopathological Society on the Standardization of Fungicidal Tests has studied the spore-germination technique and has published instructions on its use (2). Rangel (88) examined the techniques of various phytopathologists and tentatively adopted the spore-germination technique as the most suitable.

The spore-germination technique is rapid. An experiment can be set up one day and data taken the next. Aseptic precautions are required only to produce the spores. The tests can be conducted without the complications due to foreign matter inherent in agar and broth, but if foreign matter is significant, it can be

included at the behest of the experimenter, and, therefore, its effect can be measured.

The spore-germination technique is currently enjoying a wide vogue in laboratories, both State and commercial, where new fungicides are being developed. It can screen enormous numbers of new materials and it then serves admirably for quality control on the manufacture of a new material before chemical methods are devised. Oftentimes the spores can tell things about the new chemical that no other test can reveal.

The spore-germination technique is especially well adapted for work on prophylactic chemicals, but it is useful in the early stages also for therapeutic chemicals that are effective because they kill or inhibit fungi. The test is worthless, of course, where toxin inactivation is important or where prevention of sporulation is important. The technique can be used to measure both fungistatic and fungicidal power, using these words in the sense of inhibiting or of killing the spores, and as generally used, it measures fungistatic power because usually the spores are left in contact with the toxicant. This is of no serious import to strictly protective chemicals because by definition they must remain in the infection court.

Measurement of fungicidal power can be accomplished simply by removing spores after a given time from the toxicant and washing them as free of toxicant as possible. This is the standard procedure for bactericides. The bacteria in standardized numbers per volume of liquid are pipetted into tubes containing the toxicant. An aliquot is removed after suitable time intervals and plated to determine the percentage killed. McCallan and Wellman (58) have treated spores similarly, except that they centrifuged the spores out, washed them, and counted them after they germinated in distilled water.

The fungus. Fungicide researchers would laugh and sing if it were always feasible to use the organism they were interested in as a test fungus. Use of another organism causes them to worry about the possibility of differences in susceptibility (50, 61). Unfortunately it is seldom possible to use one's private fungus in the spore-germination test. *Venturia inaequalis* has received much attention in the field. It practically refuses to sporulate in the laboratory despite numerous dodges that are

said to be successful (72, 79). Sporangia of *Phytophthora infestans* are difficult to handle. Rusts refuse to grow in culture, and smuts refuse to produce chlamydospores. *Penicillium* and *Aspergillus* spores are too small to be seen readily. For these reasons two organisms at present are carrying the major load—*Sclerotinia fructicola* and *Macrosporium sarcinaeforme*. The former produces a much larger volume of spores in a shorter time than the latter, but it requires a "shot-in-the-arm" to make the spores germinate properly. Orange juice (119) is most commonly used. The latter fungus has big black spores with hyaline germ tubes, easy on the eyes to read. This becomes important to the day-by-day operator. Sometimes *Alternaria solani, Glomerella cingulata* and *Rhizopus nigricans* are used, but seldom. Only *S. fruticola* is sensitive to elemental sulfur, although the others are sensitive to organic sulfur fungicides.

Obtaining spores. Potato agar slants are suitable for producing spores of most species, although *M. sarcinaeforme* sporulates better on oat agar. Usually the spores are harvested for use as soon as the slant produces enough. About seven days are required for *Sclerotinia* and 14 days for *Macrosporium*. The spores are washed from the slants in double distilled water which obviates possible toxic contamination in the water. Some operators (63) prefer to centrifuge and wash once to get rid of nutrient in the spore suspension. That probably is necessary for the highest precision, but it is not followed in our laboratory.

Hamilton and Weaver (36) resurrected an old bacteriological dodge (75) by freezing spores in summer when they can be obtained in the field and thus preserving them for later use. This technique has merit for such desirable species as *Venturia inaequalis*.

Resistance of spores to poisoning decreases with age (10, 14); hence age should be standardized. It is interesting that house flies also become more susceptible to pyrethrum as they grow old (100).

It has been shown that the percentage of spores inhibited follows the logarithm of the dose of toxicant per spore (44, 61). Hence it is necessary to control the density of spores in the suspension. The Fuchs-Rosenthal haemacytometer (62, 120) has been recommended, but in our laboratory we find that it has

big errors because large spores, especially those of *M. sarcinaeforme,* do not act like bacteria; they are too sluggish in traveling into the counting cell, and hence the density is underestimated. It seems preferable to return to the earlier method of regulating the density by the number of spores per low power field of the microscope (10 × ocular, 16 mm. objective). Forty spores per field is a convenient number. Of course, standard procedure should be adopted in making the drop. The surface should be standard. The pipette should be standard. Uniform numbers in all drops can be obtained by bubbling air through the pipette used (80).

Spores of several species of fungi will not germinate when freed of nutrient.

It is imperative that spores germinate well. It would be eminently desirable not to have a single spore refuse to germinate because it is impossible to separate spores that are killed by the fungicide from those that are so ornery as not to germinate. Statisticians have almost tied themselves in knots trying to calculate a correction for "natural mortality", but as yet no one seems to have derived a wholly satisfactory answer (1, 24). Perhaps the greatest advantage in using *M. sarcinaeforme* is that it usually germinates almost 100%.

For those spores that do not germinate well, something must be added. McCallan and Wilcoxon (62, 63) have found that ultra-filtered orange juice from a frozen stock is suitable. Miller (71*a*) has just shown that citrates perform the same function. Sometimes biotin or coenzyme R (31) has been used. Lin (55) contended that the necessity for an added material is primarily one of energy requirement, but the work with biotin suggests that it is a vitamin shortage.

Applying spores. Drops of spore suspension are applied in duplicate to the sprayed surfaces. Of course, care should be taken to apply uniform drops, or else the dose relations will be disturbed. Drops of 0.05 ml. in volume have been standard (2). As already discussed, the drops may spread to different areas on deposits that contain wetting agents. Therefore, it may be necessary to restrict their spread with an etched ring, by using a cavity slide, or by other dodge.

The sprayed surfaces with their drops of spores are placed on

racks in large moist chambers (250 mm. D.) or so-called culture dishes. The racks may be made of glass tubing or, better still, of some metal like sheet aluminum. The chambers are inverted for use so that they can be sealed with a water seal. Since some fungicides may be slightly volatile, it is wise to have a check slide in each chamber. If volatile materials are being tried, it may be necessary to put each slide in a separate Petri dish moist chamber.

The spores are incubated usually overnight at the optimum temperature for the fungus, since the fungus is most difficult to kill at its optimum temperature (87). When germination is complete, the slides are removed and counted under low power magnification of 100 or 150×. A cover slip is not required.

The time for counting germination is more important than is usually admitted. Martin (68) pointed out that to make counts when the untreated spores are all germinated might be misleading. Later Dimond *et al.* (16) showed experimentally an influence of time, and this was confirmed by Wellman and McCallan (114). It is clear that spore germination in all treatments must be allowed to proceed to completion.

The novice usually counts more spores than necessary. Normally it is sufficient to count 50 spores in each of two drops. Since the results are influenced by natural mortality, Wellman and McCallan (114) have suggested a simple correction for spores of high viability. They suggested that as many extra spores be counted as the check is short of 100. For example, if the check gives 98% germination, then 51 spores should be counted in each drop to make a total of 102. The two are then subtracted from the ungerminated results.

The question always arises in using the spore-germination technique, whether the length of the germ tube should be determined in addition to the percentage of germination. Of course, if the length of germ tube is recorded, special statistics would be needed for evaluating the data. Hamilton *et al.* (34), without giving data, say that "the relative length of germ tubes is a better criterion of the inhibitory action of sulphur than the percentage of spores germinated". Anderson (3) made an extensive study of the two methods for measuring the inhibitory effect of wheat extracts on germination of rust spores. Apparently the two do

not bear linear relations to each other, but rather some sort of hyperbolic relation. There was no evidence that one was qualitatively different from the other. Therefore we must deduce that either is suitable but that measuring germ tubes will probably not give results qualitatively different from determining the percentage of germination. It was the opinion of the members of the Committee on Standardization of Fungicide Tests (2) that germ tube length is not of enough importance to warrant the extra work.

The Electrical Resistance Test

For many years Osterhout (78) has pursued the theory that the effect of many kinds of substances on cells can be measured by electrical resistance of the treated plants. He has confined his attentions largely to lower green plants, but one of his students (65) took a flier into fungi and fungicides. Her technique has never received much attention among the clan of fungicide researchers, perhaps because the technique seems remote from everyday experience, perhaps because she rated mercuric chloride less efficient than copper sulfate, an idea contrary to most opinion.

Growth on Agar

One of the most extensively used tests is that in which the toxicant is mixed in agar and the fungus is planted and allowed to struggle along on such poisoned food. The technique has been used mostly by those interested in wood preservatives. An extensive literature on fungicidal action has accumulated through its use.

Historically it seems significant that the spore-germination test should have been developed primarily by plant pathologists interested in protecting foliage where spores constitute the inoculum, whereas the agar test, using mycelium, has been developed by those interested in wood preservation where mycelium constitutes the inoculum.

When the two are considered side by side, it is clear, however, that the spore-germination test has great advantages in speed, accuracy and freedom from contamination by adsorptive colloidal material. It will probably therefore gradually replace the agar test where the objective is a study of fungicidal action. The agar

test will probably persist only for him who is concerned primarily about specificity of organisms and who cannot use spores of the organism concerned. He will probably reason that he would rather use his own rather than another organism, despite the disadvantages of the agar technique.

The agar test probably runs back historically about as far as solid media. It became quantitative when Falck (22) showed that the radial growth of a fungus in Petri dishes is linear with time and that the presence of a toxicant in the agar does not alter the fact. It then became possible to measure the toxicity of a compound by the number of days required to reach a particular diameter of thallus, or by the diameter of a thallus reached in a specified number of days.

The technique has reached its best performance at the Forest Products Laboratory in Madison, Wisconsin (4, 99). In brief, it involves mixing the toxicant with agar, usually malt agar, just before the agar hardens. A uniform quantity of treated media is poured into a standard Erlenmeyer flask. Upon inoculation the flask is stoppered to prevent loss of material and water by possible volatilization and to avoid cross transfer between flasks of volatile materials. The flasks are incubated at a standard temperature. Several concentrations of each material are employed. It is customary to use a standard fungus in the wood preservation studies, the commonest being an unidentified strain of *Fomes* which has a potent ability to rot wood. The new method of growing the fungus in a horizontal glass tube half filled with agar would probably be preferable to the flask method because the hyphae may be permitted to grow to greater length (93).

The agar test can be used for measuring genestatic power (12) of fungicides. If certain chemicals such as anthracene derivatives (101) permit growth but prevent sporulation, such chemicals can be listed as genestatic.

Growth on Wood Blocks or Fabrics

British and German workers on wood preservatives have bitterly opposed (23, 54) the agar technique of their American colleagues. The British have contended for the wood block method which they feel is more realistic. They inoculate a block of treated wood and incubate it several months in a Kollé flask. If the

agar technique is slow, the wood block test creeps. The wood block test is perhaps characteristic of foresters and of those concerned with wood problems because they think in terms of years whereas others think in terms of hours or days. Perhaps a wood block test is worth using as a "pilot plant" test, but for study of fungicidal action or for producing new fungicides, it is too slow for real consideration. Not only is it slow, it is not precise. Measurement of fungicidal or fungistatic action in it is not easy, and strength tests of treated blocks or gravimetric measurements of weight loss may be very difficult to evaluate. The wood block test will be discussed again under burial tests.

Waterman *et al.* (113) have defended the wood block method against the agar method because of the artificial character of the dispersion of the toxicant in agar. They have criticized the Kollé flask method, so commonly used in Europe, because the wood block becomes too saturated with water. Their technique is essentially to place the wood block in a moist chamber and to feed water to it by means of a wooden wick. Data are taken by estimating growth on the treated block, by losses in weight and by breaking tests. A similar wood block test in Petri dish moist chambers has been developed for painted surfaces by reading the growth of the *Aspergillus* indicator by a grading system (82).

The growth of organisms on treated fabrics has been used extensively in research on the use of fungicides to protect fabrics. For many years *Chaetomium globosum* was the test fungus (105). Recently species of *Aspergillus* and other genera have been used. Test strips of the treated fabric are placed in Petri dishes on a carbon-free agar base and inoculated with a spore suspension. After incubation for a fortnight, the tensile strength of the treated strips is determined. In other cases (106) no agar is used. Amount of mildew is judged visually.

Soil Burial Tests

The burial test is rapidly forging ahead as a test of protective action. It was probably first used by those interested in wood preservation, but it is currently receiving much attention by those interested in fabric preservation (5). Assay of seed protectants is a species of burial test because seeds are buried when they are planted.

The burial test is a field test in effect, and field tests are out of bounds for this paper. Nevertheless it will be discussed because at present it has advantages not now available in other tests. When treated solids or surfaces are buried underground, they come very close to an environment saturated with inoculum. Results of some sort, therefore, are assured. Whether they can be measured is another matter. If the soil is kept damp, leaching will proceed at a continuous pace, not limited to rainy or foggy periods. Organic matter is present to tie up any protein-loving fungicide, and base-exchange can proceed at a rapid pace. Of course, the type of base exchange will vary greatly with soil type and composition, and it may be unpredictable. A burial test involves the screen with perhaps the finest meshes through which a new fungicide must pass in our series of tests with new materials.

In research on wood preservatives it has been common practice to have stake farms in various areas, especially tropical areas, where treated stakes could be driven into the ground and their behavior observed at intervals. Recently the trend has been toward using much smaller blocks of wood (53) completely buried. The theory is that the smaller the block, the greater the surface area exposed to corrosion per unit weight, and hence the more rapid the destruction.

Greenhouse Tests on Foliage and Seeds

Foliage tests in the greenhouse have been used for measuring both protective and therapeutic powers of fungicides.

During the evolution of true laboratory testing of fungicides, many experimenters preferred to operate somewhere between gross field tests and the fumbling laboratory assay of the time. Keitt and his co-workers (33, 49) set up large scale greenhouse equipment complete with incubation chambers to measure protective action. The trees were sprayed with hand equipment and inoculated with pathogenic fungi. As laboratory assay became more precise it became obvious that more precision was needed in the greenhouse trials if they were to continue to compete. Hamilton and Weaver (35) designed but did not describe accurately their very elaborate and refined equipment for greenhouse assay of fungicides employing potted trees. Nielson (76) has described their equipment in somewhat more detail.

Marsh (67) sprayed leaves on twigs and placed them in a micromoist chamber after inoculation. His technique, in effect, was the same as in most laboratory assay, but he used leaves instead of an artificial surface and found that the "living leaf has proved less flattering to the fungicide than has the slide".

It is curious that McCallan, one of the most enthusiastic supporters of laboratory assay of fungicides, has now published on a method of greenhouse assay (57, 59, 60). With his colleague, Wellman, he proposed the tomato as an easily grown plant and *Alternaria solani* as a not quite so easily grown pathogene. The potted plant to be sprayed is mounted on a turntable. The "paint gun" sprayer with stirrer is mounted on the end of an adjustable arm. Plants are inoculated by atomizing them with a spore suspension at constant density. Data are taken as number of lesions per unit leaf area and expressed as percentages of the check. In another paper (115) the authors discussed the use of wheat smut in greenhouse work and also correlation between laboratory and greenhouse assays. In general, there was reasonably good agreement when it is remembered that the greenhouse assay was shown to be less reproducible than the laboratory assay. The performance of nitrogen tautomers in the greenhouse was not well predicted in the laboratory.

Salmon and his co-workers (19) at Wye in England have done some excellent research using therapy of powdery mildew on hop leaves as a "guinea pig". Hop leaves occur opposite to each other on the stem. One leaf is treated, the other is kept as check. The precision of the test is not very high, but it gave those workers some good data on the relative performance of a wide range of materials. Of course, the technique is not capable of differentiating the fungistatic from the fungicidal property of the test material.

The German workers, beginning with Riehm (91) and Gassner (29), have tested fungicides against wheat smut in the greenhouse. A couple of Ulstermen lately (73, 74) have used oat seeds infected with *Helminthosporium* and flax seed infected with one or more fungi to measure fungicidal potency. Taking heavily infected seed, they coat it with the chemical to be tested and then place 100 seeds on wet filter paper in Petri dish moist chambers. The fungus grows out through the layer of chemical over the

seed within four days if the chemical is weak. At that stage the dishes are irradiated with strong white light to induce sporulation of the fungus (*Helminthosporium*) which can be confirmed by the eleventh day. The percentage of seeds showing sporulation is a measure of toxicity of the coated layer.

Tests of fungicides on foliage and seeds will probably pay dividends until such time as the significance of the host in fungicidal action is known. The host conceivably can play a part in both the dosage and fungicidal value sections of protective value. If the surface is wrinkled, veined or hairy, it will expose much more surface for protection than a plane surface and thus influence unfavorably the dose factors, as Marsh (67) has suggested. It may aid in making the toxicant more available to the spores and hence it may activate the material, as Yarwood has suggested (122).

If the first case is true, then a simple correction for dose difference will be enough. If the second is true, then the whole assay may be disturbed in such a fashion that a simple correction will not be sufficient.

THE YARDSTICK OF FUNGICIDAL POTENCY

The potency of a fungicide is an illusive quality. It must be measured if it is to be understood, but its measurement is as shot through with fallacious thinking as any field of science. Fortunately the measurement of fungicidal potency is clearly akin to the measurement of insecticidal value, of drug value and of almost any value in biology. We can, therefore, make use of the developments in those fields. For the same reason, however, it is almost as easy to be misled by the fallacies in these other fields as it is to be helped by the progress that they have made.

The Two Methods of Measurement

Clearly in bioassay the effect of a fungicide must be measured on the fungus. Two methods of measurement suggest themselves (*a*) to measure the inhibition of growth or spore germination by any given amount of fungicide; and (*b*) to determine the amount of fungicide necessary to produce any given level of inhibition (42). Even to the casual thinker it is clear that the former method involves much less manipulation than the latter; naturally

it has been heavily leaned upon. Modern knowledge, however, shows it to be a frail reed instead of a substantial lamp post.

Measurements of response for a single dose can be made precisely enough, but they may not signify the true difference betwen any two fungicides. Moreover, the technique may sometimes rate two compounds in one order, sometimes in the reverse order (15, 109). The reasons for the anomalous situation will be developed below.

The Dosage-response Curve

If one chooses the method of comparison through the dose required for equal response, obviously he must use a series of doses of each material in each experiment and he must determine the inhibition from each dose. Such an experimental design will result in a series of data from which can be plotted a dosage-response curve. Since dose is the independent variable, it should be plotted on the X axis, and inhibition, being the dependent variable, should be plotted on the Y axis. Data so plotted on an arithmetic grid will give a sigmoid curve. The two halves of the S will not be equal, however. The upper half will be much fatter than the lower, and the upper end of the curve will tail off slowly toward the ceiling of response.

Clearly the relation betwen dose and response is flexible, but since no rubber ruler is suitable, it needs a steel center to straighten it out. If the curve were a symmetrical S, probably only one axis would need to be modified in order to straighten it. The fact that it is not symmetrical suggests that both axes need some tinkering to straighten it.

Action of dose. Reference to elementary biology or physics will recall that many processes of nature follow the law of diminishing returns which means that an increase in stimulus will not result in an equivalent increase in response. Stated in terms of the present problem, it means that an increase in response requires a geometric increase in dose. Therefore the fattened upper portion of the curve is due to the law of diminishing returns. It can be reduced to normal shape by expressing the dose in logarithms instead of in arithmetic units. This process seems to bother some assayers. It should not. They deal almost daily with pH units and these are in logarithms. Of course, they sometimes fail to

appreciate that fact because they make simple arithmetic averages of pH values.

Action of percentage response. Having eliminated the bulbous upper portion of the curve, it is apparent that the S shape must be caused by irregularities in the weight of the values on the other axis. It turns out that this is due to the rubber nature of the percentage scale. The percentage scale is least sensitive in the middle range or at 50%, and its sensitivity increases rapidly as it approaches the ceiling and the floor of response. The percentage numbers remain at equal intervals despite the fact that the sensitivity increases. It is necessary to stretch the space between the percentage values if we are to use the response scale as a firm ruler. This has been done graphically on probability paper and statistically by the use of probits which are so arranged that they measure response in equal steps. Bliss (6, 7, 8) devised the probit as a unit of measure for bioassay, but it was based on a series of earlier studies (*e.g.*, 27, 108, 117).

Having hurried thus through a maze of statistical reasoning in a few words, it can be summarized for most persons interested in bioassays by stating that data plotted on logarithmic-probability paper will give a straight line, which means that the data are now measured by a firm ruler. Data so treated provide two or three useful values, LD 50 or LD 90 and slope.

Significance of LD values. The phrase, LD values, sounds like a code message from the hero in melodrama. Actually it is shorthand for lethal dose for any given level of response. Thus LD 50 means the lethal dose for 50% response and LD 90 means lethal dose for 90% response. The derivation of these values stems back to Trevan (108). LD values are mainly a measure of fungicidal potency (16, 69), especially between samples of the material or between materials with the same mode of action. One can say with other words that LD values measure the availability factor in fungicidal value. They are wonderfully useful, for example, in quality control for new material. They tell very accurately what the relative performance of any two batches is. LD values are excellent for studying differences in particle size of any compound (69). LD values are indicative when used for two different materials, but they may be heavily influenced by the slope of the dosage-response curve, as discussed below.

Significance of slope. The angle that the straightened dosage-response curve makes with the horizontal is slope. It can be determined by calculation, but it is most easily determined by a protractor if the line is plotted on a standard logarithmic-probability grid.[1] Slope of the dosage-response curve is becoming more interesting all along as a characteristic of fungicides. It gives a dynamic picture of events. It has been made the subject of two extensive papers (16, 61). Slope can never be obtained, of course, from the static experimental design where single doses are used.

First, slope is a characteristic of the fungicide itself. Different fungicides may have different slopes. In general, slope is considered to be an indication of the mode of toxic action. If the slope differs between two compounds, it is a fair experimental assumption that they differ in mode of action. Slope, therefore, measures inherent toxicity of that portion of the material that is available (16, 69). Parker-Rhodes (81) has greatly extended the theory that slope measures inherent toxicity. He has made much of the fact that some other function of the concentration than the logarithm may be necessary to produce a linear regression line.

If, however, two materials of interest do differ in slope, then it follows that the lines must cross somewhere. If they cross, then it follows that for doses above the point of crossing, one compound will be more potent than the other, but below the point of crossing they will be reversed. Since environment probably changes the point of crossing (109), it is plain that single dose research might well show the compounds to be different in two separate experiments or in two separate areas.

Clearly in this case LD values also may be misleading. The lethal dose would be inverted for the two materials, on both sides of the crossing point. In such cases it is coming to be accepted that comparisons through some high LD value such as LD 90 are to be preferred. Such comparisons will minimize the effects of different slopes.

Recently (45), it has been discovered that slope may be a measure of coverage. The more nearly random the particles of toxicant are when exposed to the spores, the steeper the slopes will be. Therefore, if two materials seem to differ in slope, it

[1] We use grid No. 3128, made by Codex Book Co., Inc., Norwood, Mass.

would be well to ascertain whether they are exposed equally at random to the spores, or if the spores are exposed equally at random to the toxicant.

McCallan and Wellman (58) have reported that the slope of their fungistatic curve was steeper than that for the fungicidal curve for the same water-soluble chemical. This may have been due to a coverage factor in their technique. In the fungicidal technique the spores were soaked in test tubes for predetermined lengths of time in the solution of toxicant, then centrifuged and washed free of it. In the fungistatic test the liquid was placed in drops on glass slides. In both cases the number of spores per cubic centimeter was a constant. Therefore in the slide test with small drops of spore suspension, the spores probably settled onto the slide at random and were exposed almost at random to the poison. In the test tubes for the fungicidal test the spores, even at equal overall concentration per cubic centimeter, probably settled in a mass to the bottom of the tube, where each spore was clearly not exposed to as much toxicant as was each spore on the glass slide of the fungistatic test. Therefore the kill was not so high as expected. Since the position of the spores remained constant irrespective of concentration, it follows that the killing differential probably increased with concentration and the slope was flatter for a test in a tube than for a test on slides.

Slope is related to age of spores. Spores from old cultures produce flatter slopes than spores from young cultures (16). Ipsen (48) shows that the same phenomenon occurs for red cells in blood. Addition of growth promoters like orange juice (16) steepens the slope, possibly because the growth promoter may cause old spores to act more nearly like young ones.

Value of a Standard Fungicide

It is common practice in any procedure to carry a standard treatment as a reference point. In colorimetric chemical analysis, the color of the unknown is compared with that of the standard and thus the value of the unknown is determined. In toxicology also it has been common practice to refer the performance of the test toxicant to the standard.

The famous phenol coefficient so well known in bacteriology is aimed to provide such a reference comparison—phenol, of

course, being the reference. The phenol coefficient was introduced by Rideal and Walker (90). Young and Cooper (123) proposed a copper sulfate coefficient for fungicides, and later a Bordeaux coefficient was introduced for comparing protectants (44, 120).

We now know that such coefficients have been greatly overrated. They have been used in cases where they should never have been used, and they have led inevitably, therefore, to erroneous conclusions. The phenol coefficient, for instance, has been used for rating such divergent substances as nitrated derivatives, quaternary ammoniums and organic mercuries. Such a procedure is equivalent to using a copper sulfate standard in a colorimeter to measure mercury, amino acids and sodium nitrate. It is not astonishing that phenol coefficients come in for bitter denunciation at times, especially by the practical user of bactericides. Likewise, Bordeaux coefficients can be misused.

Bliss and Marks (8) have pointed out that the slopes of the dosage-response curves must be parallel if the coefficient is to be sound. Obviously if the slopes are not parallel, the coefficient will be different for each level of comparison. The explanation, of course, is still more fundamental. Since slope signifies mode of toxic action, other things being equal, it means that materials of different slope are acting differently and hence are not referable to the same standard.

The argument, like many others, breaks down if carried too far. Clearly the tensile strength of steel differs from that of lead, and copper oxide is more fungicidal than zinc oxide. These two statements in effect indicate quantitative differences of great significance to a bridge builder or a farmer. The differences can be calculated in terms of ratios or coefficients. In the case of steel it is the relative breaking strengths. In the case of fungicides it should be the relative LD 90 values, since LD 90 is high in the scale of potency but not so high as to experience too much experimental flutter towards the ceiling.

The growth of the concept of a standard can be followed from the work at Boyce Thompson Institute. At first (120) they favored the standard and a coefficient as a means of reducing day-to-day error. Later they reaffirmed their interest in the standard (63), but by 1941 they receded somewhat from their enthusiastic

position when they realized that slope made a big difference. They agree that "a standard may be employed as a check on the reproducibility of the technique and for the purpose of orientation in preliminary tests of new compounds. Likewise a standard may be used effectively to adjust the replicate test variation provided the compounds are essentially similar in chemical composition and slope" (61). In effect they agree that a standard is useful for appraising the impact of differences in resistance level (44) of spore populations from day to day. Part of their argument against the standard is that slope differences may throw it off if the comparison is at LD 50. Much of this argument fails if the coefficient is based on LD 90.

Biological Variation

The old bogey of biological variation crops up in fungicidal assays as in all similar assays. Even if all manipulative errors could be eliminated, experimenters would still be confronted with a residue of error known as the "error of sampling". This paper is no place in which to jump into the statistical intricacies of that subject, except to touch briefly on how it affects fungicidal assay.

The best technical discussions of the subject as it applies here can be found in papers by Bliss and Cattell (7), Ipsen (48) and Wilcoxon and McCallan (120). Wilcoxon and McCallan (120) have shown that most usual calculations of variation can be made graphically from the curves as plotted on logarithmic-probability paper, as already discussed. Not only can the error be determined but also Chi square can be determined from the graph by means of a nomograph which they give.

Parker-Rhodes (81) has developed a new theory of statistics for toxicity assays in which he makes use of an index of variability of spores to a toxicant. This index he calls "alpha". Correspondence and conversation with eminent statisticians in the field of toxicology reveals that no one has yet converted the implications of alpha to words of one syllable; hence it needs little more than passing mention in a general discussion.

Recently McCallan and Wellman (57, 59, 60) have made progress in statistics of using dosage-response curves on foliage diseases and spore germination.

The Threshold

One very widespread concept in toxicological research is the threshold dose for toxicity. It is a concept covered with confusion and it has therefore often impeded about as much progress as it may have fostered. Much of the befuddlement arises because the response of an individual to a poison is confused with the response of a population. Bliss and Cattell (7) speak of the threshold dose of an individual. This dose is admittedly difficult to determine experimentally and hence the "threshold" selected is admittedly artificial. Nevertheless a reasonably satisfactory judgment of threshold dose can be derived for the individual.

A spore may produce only gnarled tubes at one dose. It may be able to push out only knobs at the next higher dose and show no tube at the next. If the temperature of testing is changed to a more suitable level, these grades might be pushed up a notch so that one still higher dose is required for each response. Just which dose then is the threshold dose? One has to be chosen arbitrarily.

Still one might not wish to quibble about the threshold dose of an individual, however worthwhile it might be in precision work. For that reason the "killing point" (4) of a wood preservative on a single fungus thallus is probably defensible, although it would probably be easier to determine experimentally the dose for 90% inhibition than for 100% inhibition.

To use threshold dose for populations is a dangerous procedure because it may lead the experimenter into an indefensible position, primarily because of variation among individuals. The dose to kill the most susceptible individual is perhaps the threshold dose, but even the individual may already have been killed by "natural mortality". In practice no dosage curves are known to have been published wherein no individuals died at the weak doses. Wadley and Sullivan (110) contend for a threshold dose and yet their own curves taper off at the bottom. If there were a threshold dose for the population of flies they were using, the curve should have dropped precipitately to zero at that mythical dose. The curve did not; it merely approached zero asymptotically, just as other curves do.

Salvin (94) speaks positively of the threshold dose of an antibiotic, but his curve is smooth, tapering off towards zero with no precipitate drop to zero at the hypothetical threshold.

SOME PRACTICAL PREDICTIONS ALREADY MADE

An accelerated assay of fungicides can hardly be expected to take hold unless it "pays off". Laboratory research is paying off. It has been demonstrated (43) that a knowledge of fungicidal value and tenacity is useful in predicting the field performance of "fixed copper" fungicides. If spore-inhibiting property is equal, materials separate themselves in accordance with tenacity. If they are equal in tenacity, they separate themselves in accordance with fungicidal value. Prévost (87) made the first prediction when he decided to use copper for wheat bunt because copper killed the spores. Those interested in wood and fabric preservation have developed in the laboratory many new fungicides such as the nitrated and chlorinated phenols. Among recent fungicides for farmers that were developed in the laboratory are tetrachloroquinone (Spergon), 2-3 dichloro 1-4 naphthoquinone, disodium ethylene bisdithiocarbamate (Dithane), lauryl iso-quinolinum bromide (Q15) and phenyl mercury triethanolamine lactate (Puratized N5x).

Before many years have passed the number of new materials will be so large and so specific in their action that Bordeaux mixture and elemental sulfurs will be turned out on pasture to spend their last years in leisure as a reward for a good job well done.

SUMMARY

The field for fungicides is expanding very rapidly now with the demands for protecting military materiel from decay, and with the rise of organic compounds in the field of agriculture. Such an expanding subject demands accelerated techniques for appraisal of new developments.

With a few important exceptions in the fields of fumigation and therapy, fungicides find their major usefulness as protectants. As such they must be applied to the object to be protected before penetration has occurred. Application can be made before inoculation, as wood preservation or control of apple scab, or application can be made after inoculation but before penetration, as in the control of peach leaf curl or wheat bunt.

Protective value of a fungicide is supported on two legs—dosage and fungicidal value. Dosage concerns itself with the quantitative factors in the action, deposition, adherence of the

materal, retention by the treated surface, and tenacity or resistance to weathering. The dosage factors are, in modern parlance, the factors of logistics—to have the proper amount of material at the proper place at the proper time.

Fungicidal value concerns itself with quality factors—availability and inherent toxicity. It is not proper to speak of the fungicidal value of a material in the control of apple scab. That is its protective value. Its fungicidal value is its ability to kill the spores. Formaldehyde would have high fungicidal value, low protective value. Availability is concerned with the making of a fungicide out of an insoluble residue. It is concerned with the speed of solubility. A material with small particles is more "available" than one of the same kind with large particles.

Inherent toxicity is the ability of the toxicant once made available to kill the fungus concerned. Copper chloride has a greater inherent toxicity than zinc chloride, but copper oxide has a smaller fungicidal value than zinc chloride because copper oxide is not so available as zinc chloride.

If these factors in fungicidal action are to be appraised, techniques will have to be designed. Far and away the most satisfactory technique from most points of view is the spore-germination technique. It is rapid and it can be operated as a reasonably pure chemical system without foreign contamination, as is involved in agar. If desired, contaminations can be introduced. The method is precise within experiments and reasonably reproducible.

In general, the assay procedure for protectants with the spore-germination technique is to spray a given surface, usually a microscope slide coated with cellulose nitrate. Precautions must be taken against drifting of the spray stream in the air, against evaporation in transit and against differences in surface-tension because these may affect the size of the droplet emitted by the sprayer, the speed of travel and the coverage of the surface.

Tenacity or resistance to weathering can be assayed by giving the sprayed surface a predetermined washing.

Although it is desirable to use the fungus under consideration, it is usually necessary to use some other that sporulates and handles readily, such as *Sclerotinia fructicola* or *Macrosporium sarcinaeforme*. Precautions must be taken to take standard-aged

spores, to maintain uniformly dense spore suspensions, to regulate the diameter of spread of the spore drops, to incubate at optimum temperature for the fungus, and to permit enough time for all spores to germinate.

It is desirable to use a series of doses for each material and to determine the percentage of mortality (usually from 100 spores) for each dosage. From such data a dosage-response curve can be plotted. It will usually plot to a straight line on logarithmic-probability paper. If it does not, all elements of technique, such as volatility, diffusion and coverage, should be scrutinized before deciding that the line is really other than straight.

Such a line provides LD values and slope, that is, the lethal dose for any given level of response, such as LD 50 or LD 90, and the angle of the line. Without becoming involved in statistics unduly, it may be said that LD values measure the dose factors of quantity of deposit and also availability since availability is really a dose factor. LD values, therefore, are important in quality control of a fungicide. They show differences in particle size, *etc.*

Slope is a measure of inherent toxicity. If compounds act differently, they will show different slopes. Slope, however, may measure coverage also.

Poisoning of the food offered to a fungus is an old method of assay, and it has its advocates. Its major advantage seems to be that it permits use of the organisms of primary concern. Other than that, it is slow. It is incapable of appraising tenacity.

Some experimenters hold vigorously for using the object to be protected, such as real paint films, fabrics, wood or the growing plant. To use these slows the technique considerably. The chief advantage of using them is that the action may be different from what it is on the microscope slide. There is some evidence that the action is different. As soon as the factor causing the difference is known, however, it can be placed under scrutiny and measured by the spore-germination technique.

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THE CONTROL OF FUNGI IN LUMBER DURING AIR-SEASONING

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INTRODUCTION

Freshly sawed lumber placed in air-seasoning piles for drying is subject to the attacks of stain, mold and decay fungi until the moisture content of the wood is below the fiber-saturation point. At this point the free water in the cell lumina is gone and nearly all fungus activity ceases;[2] when thoroughly air-dry, *i.e.,* with less than 20% moisture, wood is practically immune to fungus attack so long as it remains dry. When lumber production consisted largely of old-growth virgin wood, which contained little sapwood, potential degrade of air-seasoning lumber because of fungus attack was considerably less than now. Much of the present cut is sapwood, which is particularly subject to important fungus staining and usually is more susceptible to molding and decay than is heartwood. Undoubtedly this increased proportion of sapwood is one of the main reasons why degrade caused by fungi became alarming in the 1920's, particularly in the South where much of the lumber cut is air-seasoned, and led to the studies during the early 1930's that improved control methods.

A summary of the information then available on the control of fungi in air-seasoning lumber was prepared for the National Committee on Wood Utilization in 1929 (37). Recommended control methods consisted of the use of solutions of sodium carbonates as dips, and practices to prevent log infections and to promote rapid drying in the seasoning yard. Under commercial conditions the soda dips have given uncertain protection to pine and little or no protection to hardwood lumber, and such practices as pre-steaming and end-racking of lumber, designed to give rapid initial surface drying, cannot be depended on to give adequate control under many conditions (47).

[1] Division of Forest Pathology, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture.

[2] Although the mass of evidence is that decay and stain fungi cannot develop in wood materially below the fiber-saturation point, there is some observational evidence that molds may be able to develop at slightly lower moisture contents, but proof is lacking.

Fungi causing degrade in green lumber are conveniently divided into three groups: mold, stain, decay fungi. The mold and stain fungi are mainly Ascomycetes and Fungi Imperfecti that live chiefly on cell contents. The stainers, with dark hyphae, discolor the wood; the typical molds, with hyaline hyphae for the most part, cause only surface discolorations by colored spore masses. The decay fungi are mainly Hymenomycetes and attack the cell walls. These groups are purely arbitrary and may overlap, as do some of the decay fungi which cause characteristic discoloration in the incipient stages of decay; in general, however, the important fungus species are distinguishable on the group characteristics mentioned.

These stain, mold and decay fungi exhibit wide ranges in growth rates (9, 20, 21, 28, 29, 38, 42, 44, 48, 61, 64, 77, 79) and in tolerance to chemicals (7, 8, 11, 32, 36, 40, 55, 67, 68, 74). For these reasons many of the practical problems of fungus deterioration cannot be easily studied to the best advantage in experiments with individual fungi under controlled conditions, and the main problems in the control of fungi in seasoning lumber have been studied in the field. Often these field studies have been difficult to interpret in terms of definite environmental conditions, but, on the other hand, they have yielded enormous returns in terms of commercial usefulness for the time and expense involved.

GENERAL ASPECTS OF CHEMICAL CONTROL

With certain woods, particularly those with high percentages of heartwood, and with most woods during the drier or colder seasons in some regions, provisions for rapid drying may be sufficient to secure bright air-seasoned lumber without chemical treatment (68). However, early in the work on fungus control in air-seasoning lumber it was apparent that chemical treatments must play a decisive rôle in fungus control during all seasons in such warm humid regions as the lower Mississippi Valley and in most regions during certain seasons. Up to 1930 no generally satisfactory chemicals had been found (68). The effective chemical control now in general use resulted from extensive cooperative studies by the chemical and lumber industries and the Division of Forest Pathology of the U. S. Department of Agriculture. The results of these studies appeared periodically in trade journals and were summarized in 1940 (68). Among the large number of

chemicals or combinations tested on air-seasoning lumber two groups were of outstanding success: (*a*) organic mercurials, especially ethyl mercuric phosphate and ethyl mercuric chloride at about 0.015%; and (*b*) certain chlorinated phenols at concentrations of 0.85% to 0.96%, especially sodium pentachlorphenate, sodium tetrachlorphenate and a 1–1 mixture of sodium tetrachlorphenate and sodium 2-chlor-o-phenylphenate. These chemicals best combined the qualities of effectiveness, cheapness, ease of handling and other qualities suiting them to wide commercial use. In addition to these, two previously tested chemicals, soda at about 8% and borax in saturated solution (68, 37), are still occasionally recommended for commercial use, even though the first of these has not given consistently good fungus control under severe seasoning conditions and the latter has been found consistently good only on gum (68). Satisfactory control has been secured with several other organic and inorganic materials, but various factors, mainly coloring of the wood, cost and corrosiveness to metal, have prevented their use (55, 68).

Commercially, fungicides are applied mainly by passing the lumber through a water solution of the chemical, although they are sometimes applied as sprays (68) or even by brushing. These methods afford only a surface protection during the air-seasoning period but little or no long-time prevention of decay during subsequent use.

Most of the tests on chemical control dealt primarily with the exclusion of stain fungi, although mold and decay fungi were given some consideration. As will be pointed out later, some of the chemicals recommended for general use against stains are not particularly effective against molds under severe conditions. The effectiveness of chemical dips in controlling decay fungi is more difficult to determine by visual means, but from observations on field tests (68) and laboratory toxicity tests (32) it appears that wood-destroying fungi can be adequately controlled during the seasoning period by most of the chemical dips recommended for commercial use against stain fungi. Further evidence on decay control has been obtained by tests (88) in which bright chemically treated lumber had higher strength values than stained untreated lumber, the loss in strength in the latter presumably being due partly or

mainly to decay fungi present, since stain fungi alone ordinarily do not cause material weakening of wood (68).[3]

Many of the chemicals used as general fungicides on plants or in the soil have proved ineffective in preventing fungus attack on lumber, at least in concentrations within competitive cost limits: lime-sulphur (34, 49), copper carbonate (49), Bordeaux mixture (49), colloidal sulphur (49), formaldehyde (36, 49), tetrachloro-para-benzoquinone (55, 83). In recent years there have been developed a number of surface-active detergents of high bactericidal value (14), some of which are known to be toxic to fungi (35). Among these detergents alkyl dimethyl benzyl ammonium chlorides, para-tertiary-octyl-phenyl-diethoxy-dimethyl-benzyl ammonium chloride monohydrate, and three proprietary cationic detergent disinfectants have been tested for the prevention of fungus development on seasoning lumber and found to have little or no value for this purpose (55, 83). Also, the dithiocarbamates have proved ineffective (55, 83).

THE INFLUENCE OF FUNGUS FLORAS

Because various fungi or groups of fungi show differences in their tolerance to toxic chemicals, the differences in fungus floras attacking various lumber species in various localities have a bearing on chemical control. A few species of stain fungi are widely distributed, as *Ceratostomella pini,* which has been reported from the United States (62), Japan (58), Russia (77), Great Britain (51), Germany (56) and Scandinavia (42), and *C. ips* reported from the United States (62), Japan (57) and Italy (31). However, the important stain floras vary with widely separated geographical localities (15, 31, 42, 51, 56, 76, 77, 79), with wood species (15, 77, 79), and to a less extent with season (79). Wherever extensive studies have been made, important staining has been found to be caused by several species. For a given wood species, differences in stain floras with locality have been observed in at least one instance. On *Pinus sylvestris* in Russia, *Ceratostomella coerulea* and *Endoconidiophora coerulescens* are said to be common in the Archangel Province but absent in the Leningrad

[3] Stain does cause some reduction in toughness (68), but this is not significant except for specialized uses. Recent studies (24), however, show that *Diplodia natalensis* causes more strength loss than the more common *Ceratostomella* spp.

Province (77). Such differences have not been observed in widely separated localities in southern United States (79), although during limited intervals many of the important species may be scarce, or less important species may be abnormally abundant. Therefore, if observations are for limited periods apparently important differences in stain floras on a given wood species can be found in different localities. The species of mold and decay fungi attacking seasoning lumber have not been adequately studied so that flora differences by localities or hosts in relation to control are uncertain, but it is known that there are some differences with wood species and localities.

In general, the geographical range of fungi is determined primarily by that of hosts or substrata, although in some instances other factors may be involved (6). With wood-inhabiting fungi, variations among species in cardinal temperatures for growth are sufficient to restrict the distribution, particularly locally, to certain use-conditions (9, 38, 64). The restriction of fungi by high temperatures should be less apparent in wood during the early periods of air-seasoning, since temperatures inside seasoning piles remain favorable for the development for most wood-inhabiting fungi when outside temperatures are near or above the maximum for growth of many (48). This cooling effect of evaporation is greatest during the early seasoning period, *i.e.,* the period during which fungus deterioration is greatest. During periods when the air temperatures are in the lower range for fungus activity it is conceivable that the cooling effect of evaporation might reduce the temperatures inside piles below the minimum required for certain fungi.

Physiologic specialization within the important lumber-inhabiting fungus species has not been extensively studied, and many reports of this phenomenon are based on single tests that have not been verified. Strains of *Ceratostomella pilifera* differing in temperature relations (48) and reactions to chemicals (32), strains of *C. coerulea* differing in temperature relations (48), and strains of *C. pini* differing in cultural characteristics (62) and tolerance to chemicals (32) have been reported among wood-staining species. The differences in hosts for European (42) and American (15) isolates of *Endoconidiophora coerulescens* can now be ignored, since the American isolates have been shown to comprise a distinct

species (16). Among molds from wood, forms of *Trichoderma lignorum* with widely different cellulose-dissolving ability have been found (71). Several species of wood-destroying fungi are known with forms varying in temperature relations (38), wood-destroying abilities (4, 69, 78) and pH relations (50). Judging from the common occurrence of physiologic races within fungus species in many taxonomic groups (72), it would seem likely that the knowledge of this phenomenon among wood-staining, -molding, and -destroying species is very incomplete.

The important fungi staining pine and hardwoods in southern United States are about the same on wood treated with ethyl mercuric chloride, or a mixture of sodium tetrachlorphenate and sodium 2-chlor-o-phenylphenate as on untreated wood (81). However, chemical treatments are not equally effective against all fungi. Organic mercurials permit the development of blue mold (*Penicillium*) (67) which is seldom bothersome on untreated lumber or that treated with other chemicals.[4] Treatments containing fluorides, as magnesium, sodium and zinc silicofluoride, are usually conducive to a heavy development of *Trichoderma* (55, 67). Borax appears particularly effective against wood-destroyers but oftentimes permits objectionable development of an unidentified surface-stainer (55, 68). Other cases include the tolerance of *Hormodendrum* to creosote (11), *Aspergillus* to copper sulphate (36), and a wide variety of fungi to arsenicals (7, 40, 74). The tolerance of *Hormodendrum resinae* to creosote and coal tar is particularly interesting, since this fungus not only tolerates higher concentrations than any other known fungus but apparently can grow and reproduce with no other source of nutrients than these materials. Yet this fungus is no more resistant to inorganic toxicants than a number of other fungi.

These biological differences in conjunction with varying environmental factors have made it advisable to test the effectiveness of different chemicals for each region. The organic mercurials have proved generally effective against stain fungi on all wood species wherever tested: United States (68), Finland (46, 65, 66), Great

[4] The reviewer has extensive unpublished data showing that the amount of *Penicillium* on pine sapwood treated with ethyl mercuric phosphate increases markedly, under poor drying conditions, as the concentration of the mercurial is increased from none through various concentrations up to that used commercially for stain control.

Britain (23), Malaya (53, 75), Australia (13), Philippines (7), Canada (18, 26). Likewise, sodium pentachlorphenate has proved satisfactory on all species where it has been tested: India (2), Finland (59), Gold Coast (52), Nigeria (86), United States (55, 68, 83). A 1–1 mixture of sodium tetrachlorphenate and sodium 2-chlor-o-phenylphenate has proved satisfactory in Canada (18), Great Britain (23), Rhodesia (25) and Finland (65). Other chemicals are of more limited usefulness. Sodium tetrachlorphenate alone or in combination with borax has proved satisfactory on southern hardwoods, but not on southern pines (55, 68), but is effective on conifers in the Pacific Northwest (18). Borax is effective or fairly so on *Podocarpus* in New Zealand (17), on *Dyera* in Malaya (75), and on American hardwoods but not on conifers (68).

CHEMICAL MIXTURES

Because fungi or groups of fungi differ in tolerance to some chemicals and because of the multiplicity of species of stain, mold and decay fungi that develop on seasoning lumber, treatments by single chemicals must be at relatively high concentrations in order to exclude all fungi. It is logical that the ideal treatment be a mixture of two or more chemicals from widely varying groups not only to allow protection against a wider range of organisms but also to reduce the necessary toxic concentration, thus reducing the cost and the dangers of injury to workmen. For example, ethyl mercuric phosphate is ordinarily used at the rate of 0.06 pound to 50 gallons of water, sodium pentachlorphenate at 3.5 pounds to 50 gallons, and borax at 16 pounds to 50 gallons, or as much of this as will dissolve at the prevailing temperature. If each of these concentrations is considered 100% in a scale of toxicant concentration, a mixture containing 0.008 pound of ethyl mercuric phosphate, ½ pound of sodium pentachlorphenate and six pounds of borax to 50 gallons of water would contain a toxicant concentration of about 65%. Yet this triplex mixture has proved about as effective as any of its components alone at full concentrations (55, 83). The mixture of sodium pentachlorphenate two pounds and borax three pounds per 50 gallons also has proved effective experimentally (55, 83) and is in commercial use. The advantages of such a mixture as sodium tetrachlorphenate and sodium 2-chlor-o-phenylphenate, which is in wide commercial use,

would not seem great, since the components are closely related and both cause dermatitis. The development of mixtures based on sound experimental work on toxicity seems to offer considerable promise of improving methods of chemical control of fungi. Of the mixtures so far tested the most promising for use on green lumber (55, 65) seem to be those containing borax in duplex or triplex mixtures with certain sodium chlorphenates and organic mercurials, and duplex mixtures of chlorphenates and organic mercurials. However, the most advantageous proportions of the various components have not been determined. Also, other factors need attention before low concentration mixtures can be recommended for commercial use. Does the borax maintain sufficient alkalinity of the treated wood surfaces to prevent conversion of the soluble phenate to the insoluble phenol, thus increasing the chance of loss by rain wash? Solutions of the ethyl mercuries are known to lose toxicity with use (26), probably through selective adsorption of the mercury by wood fibers. Consequently, this may seriously reduce the toxicity of solutions containing a low initial mercury content.

No studies on possible synergistic effects on wood-inhabiting fungi have been encountered. Such effects might occur but could not be detected in field studies in which wood is attacked by many species of fungi. It is more reasonable to assume that the effectiveness of mixtures on air-seasoning lumber is due more to the various components being effective against different fungus species than to synergism.

EFFECT OF THE SEASON OF TIMBER FELLING ON RESISTANCE TO FUNGUS ATTACK

It has long been believed by wood users that wood felled in the winter is more resistant to fungus attack than that felled during the growing season. Timber cut, milled and placed in air-seasoning piles during the winter usually has a lower surface moisture content at the advent of weather warm enough for rapid fungus infection. Limited and rather inconclusive experimental data from Sweden (42) indicate no consistent differences in susceptibility of winter- and spring-felled spruce and pine to blue-stain fungi under laboratory conditions. Field tests show that severe lumber staining and molding can occur in southern United States

during both winter and summer, weather conditions permitting (10, 55, 83).

The only extensive researches on the effect of season of felling on the susceptibility of wood to fungus activity are those of Gäumann (27, 28) who, from laboratory and field tests in Switzerland, concluded that spruce and fir felled during the period of active stem growth was at least twice as susceptible to decay as that cut during the winter. The differences, however, were of little practical significance in the wood that had been fully seasoned. With beech the seasonal differences found were not of practical magnitude. Although these data are outwardly convincing and have been generally accepted by reviewers, they should be confirmed by further tests before being accepted. Doubt is raised because the seasonal differences in susceptibility to decay were reported for the relatively inert heartwood as well as the sapwood, and the sharp rise in decay susceptibility started with the felling at the middle of February, before one would expect much growth activity. Only under the most completely controlled conditions is it possible to get reliable comparative data, even when all samples are tested simultaneously. With tests started during different seasons, and each extending over a period of several months, it would be extremely difficult to maintain uniform conditions, particularly moisture content of the test sample. Certainly Gäumann's researches bring out interesting possibilities, and it would seem distinctly worth while that further work on the effect of time of felling on durability be done, particularly with some of the more decay-resistant woods as white oak and bald cypress.

NECESSARY ADJUNCTS TO CHEMICAL CONTROL

There is ample evidence based on experimentation and observations under commercial conditions that the use of chemicals on green lumber in itself will not always insure adequate control of fungi during the subsequent air-seasoning period. The short period dips or sprays used in treating green lumber are relatively superficial and cannot be expected to kill previous infections deep in the wood or to be effective over protracted periods. Therefore, chemical treatments must be supplemented by handling methods that permit treatment before infections become deep-seated and that permit rapid drying after the chemical is applied.

Quick Utilization of Logs. Deep-seated infections of logs prior to sawing into lumber raise one of the most serious problems in the control of fungi in seasoning lumber (47, 80, 82). In cold weather or in logs that are sawed soon after cutting, log infections are seldom bothersome. The length of time fresh logs can be safely stored out of water varies so much with locality, season, wood species and other conditions that no general recommendations can be made. Spraying logs with fungicides or the use of appropriate end-coatings are effective means of preventing fungus infections so long as insect attack is not prevalent (41, 68, 73, 91). The fungicides in use show no deterrent effect on bark and wood-boring beetles (52, 68, 82) that are effective agents in inoculating through the protective chemical shell. The constant association of certain fungi, including staining species of *Ceratostomella,* and bark and ambrosia beetles is fully established (19, 43, 62, 63, 64, 82, 84, 89, 90). Inoculations by beetles often result in infection of standing trees and logs. From the point of view of log protection there is a need for cheaper methods of beetle control. Recent studies (12) show that chemical control of beetles is possible, but the really effective chemicals are rather costly. Storage of logs in water is also an effective method of preventing both fungus and beetle damage (5, 42) provided the entire log is kept wet. In southern United States, at least, water storage is much less common than in the past because the present logs, with a greater sapwood content, are higher in specific gravity and sink so soon that raising them increases handling costs excessively.

Although one of the main purposes of water storage is to maintain during the storage period conditions unfavorable for fungus and insect development, there is also the possibility that water storage may have an influence on the susceptibility of wood to fungus attack after sawing into lumber. The available data are not conclusive, but they indicate that the effect of water storage on subsequent fungus activity would vary with fungus and wood species. Preliminary studies indicate that prolonged water storage of coniferous logs reduces the susceptibility of lumber cut from them to attack by some stain fungi (1, 42) but not by others (42). Water-stored coniferous logs seem more susceptible to attack by the mold *Trichoderma* (22), which may render the wood less suitable for other fungi, possibly because of toxins produced.

Water storage also tends to deplete stored carbohydrates and thus reduces attack by some beetles (60, 87) and perhaps certain fungi (87). In contrast, should the wood be one naturally resistant to fungi because of water-soluble toxic extractives (33), water storage might sufficiently reduce these to render the wood more susceptible to attack (70); should the extractives not be especially water-soluble, water storage for usual periods would not be expected to decrease durability greatly (54). Furthermore, storage in sea water or in pond water loaded with extractives from previous lots of logs might give results different from fresh water.

In northern European literature there are records of tests of novel methods of treating or storing logs to prevent fungus attack: natural resin impregnation (39), continuous water sprays (45), and covering log piles with a thick layer of coniferous boughs (42). For economic or climatic reasons these would not seem feasible under many American conditions. Also, it seems doubtful that some of the methods described are in fact effective, since the evidence is conflicting (42, 77).

Minimum Delay Between Milling and Chemical Treatment. Lumber leaving a sawmill is likely to be heavily inoculated with fungi (82), and chemical treatment must be done before these inoculations develop into deep-seated infections. The rate of growth of fungi, particularly the rate at which they penetrate wood, has an important bearing on this problem. Different important stain fungi have widely varying growth rates, ranging from about 2 to 20 mm. in radius per day on agar media at 25° to 30° C. (42, 48, 79), which is near the optimum temperature for many stain and decay fungi. The rate at which *Ceratostomella pilifera* penetrates pine wood tangentially, radially and longitudinally, approximates the ratio 1:2:9, the longitudinal penetration being about equal to the radial growth on agar under favorable growth conditions (48). The same fungus under favorable laboratory conditions penetrated pine wood sufficiently far in 48 hours to escape killing by a 10-second dip in ethyl mercuric chloride; boards dipped 24 and 12 hours after inoculation developed but slight interior stain. European studies on penetration of stain fungi (42), although not easily interpreted in relation to chemical control, indicate that chemical treatments 24 to 48 hours after sawing would kill most stain-fungus infections. Assuming that the corre-

lation between growth on agar and penetration in wood found for *C. pilifera* (48) holds for other stain fungi, some of the faster growing species, as *Diplodia natalensis,* with a growth rate on agar of four to five times that for *C. pilifera* (79), might under ideal conditions penetrate wood sufficiently far in 12 hours to escape killing by subsequent chemical treatment. However, field tests (68) show that under usual commercial conditions satisfactory control can be secured if lumber is dipped within 24 hours from the saw, provided there are no deep-seated log infections prior to milling. During cool weather longer periods of delay probably would be safe.

Although this recommendation is made for the control of stain fungi, it should be applicable to mold and decay fungi also. Most molds cause degrade mainly by the presence of colored spores on the wood surface (68). Those molds studied, however, have been found to penetrate wood (30, 67, 71, 85). *Trichoderma lignorum* appears to penetrate pine wood more slowly than does *C. pilifera* (68, 71). Also, molds are of less economic importance, the visible evidence of them being removed by the usual surface planing or by a mechanical brush treatment (3). No information is available on the rate of penetration of decay fungi in seasoning lumber. On agar a large number of decay fungi grow at rates (9, 38) about equal to the stainers of intermediate growth rate (42, 79), and with none approaching the growth rates of the faster growing stainers.

Reducing Bulk-Piling Periods to a Minimum. In lumber handling practices it is sometimes necessary or desirable to put treated lumber in solid or bulk piles for varying periods before it is placed in regular, ventilated air-seasoning piles. The effectiveness of the commercial fungus-control chemicals in protecting lumber during bulking periods beyond seven weeks is doubtful under the severe conditions of southern United States (68), but much longer bulking periods seem safe in the Pacific Northwest (18), particularly if higher than normal concentrations of the chemical are used. However, there is only fragmentary evidence on how much fungus activity will subsequently occur in air-seasoning piles after the bulking period (68). Further information is needed before positive conclusions can be drawn.

Good Air-Drying Conditions. To insure good stain control by

chemical treatments it is necessary to have rapid drying provided in the seasoning yard by good soil drainage, weed control, and arrangement and construction of piles to facilitate air movement; and to protect the treated lumber from rain wash both before and after piling (68). The experimental evidence for these statements is rather meager, but from theoretical considerations, experience with chemicals under severe conditions in small scale tests (55, 68, 83), and extensive observations in commercial yards there is little doubt that provisions for rapid drying are necessary.

Avoidance of accumulations of wood refuse in and around seasoning yards is usually listed as a desirable practice in stain control (37, 68, 80), although there is no direct evidence for this. General observations over a period of years indicate that the deleterious effect of such accumulations would, under most conditions, be limited to the effect of obstructing air movements, and that an abundance of inoculum is present even in those yards with the least wood refuse. There is some experimental evidence supporting this (82).

CONCLUSIONS AND SUMMARY

The control of mold, stain and decay fungi in lumber during air seasoning has become progressively more important as the amount of the more fungus-susceptible sapwood increases with the passing of virgin timber stands and as consumer demands become more critical. This control is best accomplished by the use of chemical dips in conjunction with practices that prevent deep-seated log infections, that permit quick chemical treatment after sawing into lumber and that promote rapid drying in the seasoning yard. It is estimated that under peacetime conditions as much as $3\frac{1}{2}$ billion board feet of lumber annually are dipped in chemicals for the prevention of fungus deterioration in seasoning yards.[5]

Some of the chemicals used on green lumber have been found satisfactory on all species of wood and in all regions where tested; others are satisfactory only on certain woods, particularly in certain regions. Some are not effective against molds, although highly effective against stain and decay fungi. Because green lumber is attacked by large numbers of fungi with varying tolerances toward certain chemicals, single-chemical treatments are usually applied at

[5] Based on informal reports of the sale of anti-stain chemicals.

relatively high concentrations to afford an adequate factor of safety. The most recent tests on the control of fungi in green lumber show that mixtures of chemicals offer promise of improving control by reducing the total toxic concentration needed, thus reducing the cost and dangers of injury to workmen. This is presumably due mainly to giving protection against a wider range of fungi. More work is needed on the most advantageous mixtures; the effect on the solution of selective adsorption by the wood, particularly when the chemicals are used in dilute concentrations; and the effect of rainwash on mixtures containing borax.

Of the factors influencing the effectiveness of chemicals on green lumber one of the most bothersome is that of deep-seated infections in logs prior to sawing into lumber. Much of this infection could be prevented by proper integration of woods and milling operations to prevent long-time log storage. Since this is not always feasible there is a need for more effective chemicals for use on logs. Effective and cheap fungicides are available, but they do not repel bark and ambrosia beetles that penetrate the logs and inoculate the wood inside the protective chemical shell. The development of effective and cheap beetle repellents is needed.

In seasoning lumber the actual mechanics of fungus control are better known than many of the biological factors involved. The mills that follow recommended practices generally get bright lumber at moderate cost. Yet the reason why certain chemicals (as borax) are effective only on hardwoods or others (as sodium tetrachlorphenate) on hardwoods only in one region, but on both hardwoods and conifers in another region, is not certain. Perhaps it is merely a matter of differences in fungus floras, although chemical or physical chemical reactions between the fungicide and the different woods may be a factor. More information is needed on fungus floras on different woods in different regions, particularly those associated with failures of chemicals. This information would afford a better basis for devising chemical mixtures.

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Specialization, Hybridization and Mutation in the Cereal Rusts	T. JOHNSON and MARGARET NEWTON *Dominion Laboratory of Plant Pathology*
Preventing Plant Disease Introduction	W. A. MCCUBBIN *U. S. Bureau of Entomology and Plant Quarantine*
Aerobiology in Relation to Plant Disease	E. C. STAKMAN and CLYDE M. CHRISTENSEN *University of Minnesota*
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Articles in course of preparation

The Cytology of Fertilization in Angiosperms	L. E. ANDERSON *Duke University*
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